Physiological and molecular characterization of active fungi in pesticides contaminated soils for degradation of glyphosate

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Abstract

Understanding the physiological and molecular characteristics of naturally occurring fungi in glyphosate pesticide-contaminated environment is crucial to managing its contamination. The study was aimed at isolating and characterizing soil fungi for their physiological roles towards glyphosate degradation. Pure cultures of fungi were isolated from soil contaminated with glyphosate at farms in Lagos, Nigeria. The cultures were grown on minimal salt agar media amended with glyphosate. The best isolates exhibiting good tolerance to the glyphosate were characterized using molecular techniques. The BLAST search indicated that the fungi belong to four Aspergillus species (*Aspergillus flavus* strain JN-YG-3-5, *Aspergillus niger* strain APBSDSF96, *Aspergillus fumigatus* strain FJAT-31052 and *Aspergillus flavus* strain APBSWTPF130, *Trichoderma gamsii* and *Penicillium simplicissimum*. The biodegradation study of the glyphosate by the selected fungi species showed the presence of Aminomethylphosphonic Acid (AMPA) except for *Aspergillus fumigatus* strain FJAT-31052. Annotation analysis of the partial gene sequence showed that the strains possess protein coding gene clusters for glyphosate utilization and other physiological activities. The GhostKOALA output confirmed that CYP2W1 gene (Cytochrome P450, fungi type) was present in *Aspergillus fumigatus* strain FJAT-31052 which was absent in the genome of other fungi. The physiological and molecular characteristics of *Aspergillus fumigatus* strain FJAT-31052 clearly show that this fungus is a useful organism for managing contamination by glyphosate pesticide.

Introduction

Glyphosate (N-(phosphonomethyl) glycine) pesticide is a widely used pesticide against a broad spectrum of pests such as weeds, insects, fungi, nematodes and rodents in agriculture [1, 2]. The intensive use of pesticides has led to an increased level and risk of contamination of the ecosystem and harmful effects on biodiversity, food security, water and other resources [3, 4, 5]. Glyphosate is a highly effective pesticide because it prevents the biosynthesis of some notable proteins that are needed for plant growth and it also inhibits a specific enzyme pathway known as the shikimic acid pathway. This pathway is important for plants and some microorganisms. Studies have shown that the inhibitors of the shikimate pathway enzymes are potential herbicidal because an inhibitor of a key enzyme of plant metabolism might be herbicidal without being toxic to animals. Also, this pathway operates only in plants and microorganisms [6].

Glyphosate is highly soluble in water. The high solubility in water and strong binding capacity to soil organic matter by glyphosate is the reason for its fast and easy distribution in ecosystems compartments [7]. The half-life of glyphosate ranges from 8.3 to 141.9 days, and it has been reported to be up to 1 year in some extreme cases [8]. The differences in rates of glyphosate degradation might be due to the changing microbial activity and extent of soil-binding. The ability of microorganisms to degrade glyphosate assumes the occurrence of enzymes cleavages by utilization of glyphosate as sources of energy. According to Sviridov *et al*. [9], two pathways have been proposed for glyphosate degradation, which is the AMPA pathway and the C-P lyase pathway. In the AMPA pathway, glyphosate is cleaved into aminomethylphosphonic acid (AMPA) and glyoxylate by the presence of glyphosate oxidoreductase, whereas in the C-P lyase pathway, degradation of glyphosate is catalyzed by C-P lyase with the formation of sarcosine as an intermediate product, which in the end forms formaldehyde and glycine in a reaction catalyzed by sarcosine oxidase [9].

Microorganisms known to degrade glyphosate by way of glycine comprise *Arthrobacter sp*. strain GLP-1 and *Pseudomonas sp*. (strain PG2982) [10]. Research has also shown that the cleavage of the C-P bond of glyphosate to produce sarcosine and finally to glycine is mediated by sarcosine oxidase-dehydrogenase [11-13]. It has also been reported that some groups of bacteria, represented by a *Flavobacterium sp*. (Strain GDI) as well as the earlier-reported mixed bacterial cultures from soil degrade glyphosate by cleaving its carboxymethyl carbon-nitrogen bond to produce AMPA [10]. Some of the AMPA generated in this way if not further metabolized is a great concern to the environment because of their potential toxicity. This has led to the mounting concern of extensive contamination of the environment resulting in likely potential risks to non-target organisms due to entry into the food chain.

Despite the imminent toxicity posed by environmental threats, some organisms can still withstand glyphosate. The ability of some organisms to survive and live in a polluted environment and remediate it depends on physiological, molecular, genetic and ecological traits possessed by such organisms. Fungi are extremely important for many physiological functions in environmental assessment and protection. Their physiological and molecular determination can be useful in ecotoxicity studies and ecosystem management. Based on this, we selected two farms in Lagos Nigeria where glyphosate is used as herbicides, isolated the fungi in pure culture, grow them in the medium using glyphosate as energy and study their physiological and molecular roles towards glyphosate degradation.

Materials And Methods

2.1. Soil samples collection

The soil samples used for this study were obtained from two farms: Abeto and Igbalu Farms in Ikorodu, Lagos, Nigeria. The selected farms have a history glyphosate organophosphorus herbicide for the past 5-6 years to control pests. Glyphosate is effectually still used to control pests at the farms. Soil samples were collected from four different points in each farm's location at 100 metres apart as described by Asef [14]. The soils were collected using a spatula at a depth of 15 cm and transferred to sterile containers. The soil samples were transported to the laboratory and stored at 4 °C until further analysis. Soil samples were air-dried 24 h and sieved through a 10 mm mesh before the screening.

2.2 Quantification of glyphosate content and metabolites

Pesticides and their metabolites were measured and quantified using Gas chromatography as described by Moye and Deyrup [15]. Samples (about 2.0 g) were extracted with 10 mL of an aqueous solution containing 0.25 M NH_4OH and 0.1 M KH_2PO4. The samples containing the aqueous solution were shaken on a mechanical shaker for 60 m. A 2-mL aliquot of the supernatant was withdrawn using a 0.45 µm syringe filter. This extract was stored in GC vials, before being derivatized. Aliquots (1.6 mL) of the derivatization reagent mixture (by mixing 1 volume of Heptafluoro butanol (HFB) to 2 volumes of Trifluoroacetic

anhydride (TFAA) were added to 2 mL GC vials. The vials were sealed using screw caps with septa. The vials were then cooled to about -4 °C before proceeding. A variable volume Eppendorf pipet was used to add a 36 µL aliquot of sample extract (or a dilution of the extracts), or standard solution to the derivatizing reagent. Analyte derivatization was then performed by heating the reaction vials to 95 °C for 1 h and then cooled to room temperature. The excess derivatization reagents were evaporated under a gentle stream of nitrogen. The residue was dissolved in 200 µL of ethyl acetate, capped and stored for Gas Chromatographic (GC) analysis.

Chromatographic analysis was performed using an Agilent GC/MS, equipped with a split/splitless injector, and an autosampler. The analytes (glyphosate and AMPA) were quantitated using the standard method with calculations based on peak area. Analyte quantitation was carried out with Agilent Mass-Hunter Gas Chromatographic (GC) software [15]. Also, aminomethylphosphonic acid (AMPA), a primary degradation product of glyphosate in the samples were assayed using the method of Ermakova *et al.* [16]. Glyphosate calibration stock solution was made by diluting aliquots of the herbicide concentrate to known amounts of the analyte in water. Working calibration solutions of 1, 10 and 100 µg mL^{-1} were made by serially diluting the stock solution as needed.

2.3 Soil incubations, isolation and colony characteristics

The isolation of glyphosate degraders from soil samples was done to screen for strains that could degrade glyphosate in a liquid enrichment medium. Ten grams of soil sample was weighed on an analytical balance. One gram of the sample was transferred into 90 mL of sterile Minimum Salt Medium (MSM) broth in a 250 mL of Erlenmeyer flask respectively and incubated at 30 °C in a gyratory shaker for seven days. After the incubation period, 1.0 mL of sample was withdrawn and was serially diluted using 9 mL of sterile distilled water up to 10^5 dilutions. All the replicates of each soil type and treatment were treated separately for isolation purposes. Pesticide degraders were enriched in the dark on a shaker at 180 rpm for 7 d. The colony characteristics were examined. A second enrichment was done thereafter as described below by transferring pre-grown culture from each of the replicates to the fresh media.

2.4 Enrichment of glyphosate degraders

The fungi isolated from the soil samples were afterward used for the enrichment of potential glyphosate degraders. These media were further treated separately with glyphosate to enrich for glyphosate degraders. The enrichment of glyphosate degraders was carried out in liquid media by dissolving the components in 1000 mL of distilled water and adjusting the pH of the basal medium to 6.0 using 1 M NaOH solution. 150 mL of the basal medium was dispensed into 250 mL Erlenmeyer flask and the pesticide substrate was introduced into each flask at 100 ppm after sterilization in an autoclave at 121 °C for 15 m. 1.0 mL aliquot of diluted broth culture of each isolate (10^4 cells mL^{-1}) were seeded into each flask and incubated in a gyratory shaker incubator at 150 rev/m at 30 °C for a period of 32 d. The growth and enrichment ability were monitored at 4 d intervals. The utilization of the pesticide fractions by the fungal isolates was evaluated by monitoring the fungal growth measured by viable count on PDA, the Optical Density (OD) at 620nm wavelength with 770 UV/Visible Light PG Spectrophotometer and changes in ionic concentration pH was determined with pH meter (model P2II) [17].

2.5 Degradation of glyphosate

The medium was supplemented with glyphosate (100 ppm). The fungal isolates were first grown in the medium supplemented with glyphosate to determine activity of fungi in the degradation of glyphosate.

The percentage loss of the pesticide was calculated as: **see formula 1 in the supplementary files.**

While the efficiency of biodegradation was calculated using the formulae: LT – LC (Where LT is % loss of pesticides in treatments and LC is % loss of pesticides in control).

2.6 Molecular characterization of isolates

2.6.1 Colony DNA extraction and amplification by polymerase chain reaction (PCR)

Genomic DNA was extracted from the isolates using a Zymo Quick-DNA Fungal Micro-prep kit. After extraction, the DNA concentration and purity were checked using a ThermoScientific Nanodrop, model 2000. The extracted DNA were amplified with primers for genes used for identifying fungi. The Internal Transcribed Spacer (ITS) gene for characterization of fungi, ITS universal primer set which flanks the ITS4, 5.8S and ITS5 region was used:

- ITS4 TCCTCCGCTTATTGACATGS
- ITS5 GGAACTAAAAGTCGTAACAAGG

Polymerase chain reaction (PCR) conditions were set on an initial denaturation temperature of 94 ˚C for 5 m, followed by 35 cycles of 30 s denaturation at 94 ˚C, 30 s annealing of primer at 55 ˚C, 1½ minutes extension at 72 °C and a final extension for 7 m at 72 °C [18].

The amplified fragments were purified by ethanol in order to remove the PCR reagents, before they were sequenced using Applied Biosystems Genetic Analyzer 3130xl sequencer and Big Dye terminator v3.1 cycle sequencing kit. Bio-Edit and MEGA 6 were used for all genetic analysis.

The classification/identification of organisms were performed by a local nucleotide BLAST search against the non-redundant version of the NCBI reference database [19]. Phylogenetic relationship analysis was performed on the sequences of isolated microorganisms using Molecular Evolutionary Genetics Analysis version 6 [19]. The sequences were prepared using FASTA format and aligned using ClustaW option of the program [19]. The phylogenetic dendrogram was constructed using the maximum likelihood with 1000 bootstrap.

2.6.2 Genome sequence annotation

The annotation of protein-coding genes was provided by FGENESB; further gene prediction and functional annotation were performed by GhostKOALA. The pathways of selective compounds were interpreted using KEGG pathway chart.

2.7 Statistical analysis

Data sets were analyzed using origin software and GraphPad prism 8.04. Comparative of means were done using descriptive statistics. Significant means were compared using Tukey's multiple T-test and two-way analysis of variance (ANOVA). All graphical presentations were done using origin software. Correlation analysis was done to compare the relationship between pesticide degradation, colony count, optical density and pH. Biodegradation study of glyphosate was expressed as percentages, evolution of AMPA and glyphosate and as co-efficient of degradation using descriptive statistics. Molecular identification and characteristics were done using e-values and percentage identity.

Results

3.1 Characterization of the soil from the locations

All the locations had evidence of glyphosate contamination though the contamination levels varied depending on location (Fig. 1) and they were significant ($p<0.05$). Location 1 had the highest contamination comprising 319.1 ±0.01 mg kg^{-1} glyphosate and 194.2±0.01 mg kg^{-1} AMPA. Location 3 showed increased glyphosate level without transformation product (AMPA). This could suggest the active state of the organisms' present. Among the locations, the lower level of glyphosate was observed more rapidly in location 4 with only traces of glyphosate present (6.98±0.00 mg kg^{-1}).

Correspondingly, the fungal density varied within the location (Fig. 2). There was a high enumeration of fungal count in locations where AMPA concentration was high and with significant concentrations of glyphosate. However, other locations where AMPA concentration was low or absent had low fungal count. This implies an active metabolic state of the fungi. As a result, this study evaluates the relationship between the fungal density, glyphosate and AMPA. The Pearson correlation shows that fungal load is significantly related to AMPA ($r=0.94965$; $p\leq0.05$) in comparison to glyphosate.

3.2 Selection and isolation of glyphosate degraders

A total of 14 isolates were obtained from the farms (Table 1). The fungal inoculum from soils enrichment was plated on MSM agar plates to check for their ability to grow in the presence of glyphosate on solid media. It was observed that S1b, S1c, S2.3, S3.2, S3.3, S4.1 and S4.4 isolates did not grow from any of the enriched soils on MSM agar when glyphosate was added externally, during the set incubation time. They displayed poor (+) clear zone; therefore, no further analysis was conducted on them. The isolates that showed good (++) clear zone (S1a, S1d, S2.1, S3.1, S4.1 and S4.3) were further analyzed for their ability to degrade glyphosate. A total of six (6) potential degraders were obtained after successive sub-culturing from the soils (Table 2).

Table 1 Summary of the fungal culture characteristics

Isolate code	Colour/pigment	Growth of Isolate on AGAR+ Herbicide	Optical Density at 620nm		
Day 0	**Day 4**	**Day 7**			
S1a	Greenish Yellow	++	0.056	0.261	0.569
S1b	White	+	0.034	0.133	0.166
S1c	Green	+	0.042	0.158	0.386
S1d	Black	++	0.063	0.246	0.399
S2.1	Yellow	++	0.052	0.258	0.421
S2.2	Brown	++	0.076	0.274	0.481
S2.3	Green	+	0.039	0.144	0.383
S3.1	Green	++	0.048	0.261	0.493
S3.2	Green	+	0.035	0.147	0.171
S3.3	White	+	0.041	0.166	0.352
S4.1	Green	++	0.059	0.241	0.581
S4.2	Grayish Green	+	0.033	0.193	0.236
S4.3	White	++	0.038	0.186	0.311
S4.4	Brown	+	0.061	0.263	0.461

Key: + poor clear zone, ++ good clear zone; S1 – Soil 1; Sla, S1b and Slc (1^{st}, 2^{nd} and 3^{rd} isolates from soil 1); S2 – Soil 2; S2.1, S2.2 and S2.3 (1st, 2nd and 3rd isolates from soil 2); S3 – Soil 3; S3.1, S3.2 and S3.3 (1st, 2nd and 3rd isolates from soil 3); S4 – Soil 4; S4.1, S4.2, S4.3 and S4.4 (1st, 2^{nd}, 3^{rd} and 4th isolates from soil 4).

Table 2 The selected isolates, locations and identities

Selected isolate	Source	Organisms Name
S1a	Location 1	*Trichoderma gamsii* P2-18
S1d	Location 1	*Aspergillus flavusJN-YG-3-5*
S2.1	Location 2	*Aspergillus niger* APBSDSF96
S3.1	Location 3	*Aspergillus fumigatus* FJAT-31052
S4.1	Location 4	*Aspergillus flavus* EFB01
S4.3	Location 4	*Penicillium simplicissimum* SNB-VECD11G

3.3 Enrichment of glyphosate degraders

We use optical density (OD) at a wavelength of 600nm (OD_{600}) in addition to colony-forming units (cfu) to evaluate microbial enrichment and growth in liquid fungal culture (Fig. 3). Several distinct growth phases were observed within the fungal growth curve, and these were the lag phase, the exponential or log phase, the stationary phase, and the death phase. Each of these phases represents a distinct period of growth that is associated with typical physiological changes in the cell culture.

Six fungal isolates were stimulated to grow in the presence of glyphosate. The glyphosate mixed with MSM showed enhanced growth of the fungal isolates as shown by their optical density and cfu. The logarithmic growth phase for OD_{600} lasted for 8 d in all the fungal isolates while for cfu, it lasted for 24 d. The stationary phase commenced after day 8 in growth curves for OD_{600} till the end of the experiment whereas the cfu had a very sharp stationary phase from 24 d to 28 d. The exponential growth (log phase) for cfu was observed to be biphasic with a transition from an initial to a subsequently slower rate of growth leading to the stationary phase. The first exponential phase was between 8 d and 12 d, while the second exponential phase was between 16 d and 24 d. The OD_{600} growth did not have the death phase while cfu had a death phase after the 28^{th} d of incubation until the end of the experiment. The discrepancy between cell density by the form of cfu and OD600 could be from the hypothesis that optical density measurement is the quantity of viable and non-viable cells in a sample. The colony-forming unit measures only the viable cells in a sample. An OD 600nm is an approximation CFU/ml and cannot fully take into account the non-viable cells the OD may be reading. It may also depend on the microorganism that is being researched.

Consequently, the specific growth estimated as a function of first-order kinetics revealed *Trichoderma gamsii* P2-18, *Aspergillus flavus* JN-YG-3-5, *A. niger* APBSDSF96, *A. fumigatus* FJAT-31052, *A. flavus* EFB01 and *Penicillium simplicissimum* SNB-VECD11G have 0.47, 0.40, 0.57, 0.49, 0.41 and 0.46 cfu/day respectively. From these findings, the growth of fungal isolates, *A. fumigatus* FJAT-31052 and *A. flavus* EFB01 were promoted and can grow in glyphosate whereas, *T. gamsii* P2-18 had the lowest growth promotion compared to other fungal isolates. Hence, specific growth values at the two selected points were calculated for both phases of growth. The data shows that the specific growth rates are comparable for the two different exponential phases. From these findings, the order of the growth of the isolates as estimated from the slope of the growth curve in cfu are as follows: *P. simplicissimum SNB-VECD11G* > *A.fumigatusFJAT-31052* > *A. flavus EFB01* > *A. niger APBSDSF96* > *A. flavus JN-YG-3-5* > *T. gamsii P2-18* (0.23 cfu/day, 0.2282 cfu/day, 0.2257 cfu/day, 0.2234 cfu/day, 0.2195 cfu/day and 0.2171 cfu/day) respectively. The growth of fungal isolates, *A. fumigatus* FJAT-31052 and *A. flavus* EFB01 were more promoted in glyphosate compared to others whereas, *T. gamsii* P2-18 had the lowest growth promotion. Therefore, we hypothesize that these isolates' biodegradable ability to glyphosate will differ.

In this study, the initial pH for all the isolates was weakly acidic (within 6.0) (Table 3). Thereafter it declined gradually and became more acidic at the end of the experiment. The change in pH was more obvious in *A. flavus* EFB01 (22.06%) and lowest in *A. flavusJ*N-YG-3-5 (19.21%). From this, we can hypothesize that fungal growth is inversely proportional with the pH. This suggests that fungal are more active in a slightly acidic environment compared to a basic environment.

Table 3 Summary of pH changes with respect to days

Sample code	Day 0	Day 4	Day 8	Day 12	Day 20	Day 24	Day 28	Day 32	% change in pH
T. gamsii P2-18	6.06	6.00	5.00****	5.76	5.30**	5.00****	4.86****	4.75****	21.62
A. flavusJN-YG-3-5	6.04	5.96	5.80	5.66	5.44	5.24**	5.06***	4.88****	19.21
A. niger APBSDSF96	6.06	5.99	5.84	5.74	5.33 **	5.16 ***	4.92****	4.79****	20.96
*A.fumigatus*FJAT-31052	6.05	6.01	5.81	5.63	5.22**	5.03****	4.89****	4.73****	21.82
A. flavus EFB01	6.03	5.96	5.82	5.50	5.10***	4.96****	4.83****	4.70****	22.06
P. simplicissimum SNB-VECD11G	6.04	5.95	5.78	5.46	5.18****	5.00****	4.90****	4.82****	20.20

Values with asterisk have significant difference between the day of observation and day 0. * = p<0.05; **= p<0.001; ***= p<0.0001; ****= p<0.0000. % change in pH is from difference between day 0 vs day 32

3.4 Degradation of glyphosate

The potential ability of the six fungal strains for glyphosate biodegradation was observed for 32 days (Fig. 4). Our hypothesis is that these selected isolates' ability to degrade glyphosate will differ because their growth in glyphosate differed. The strain *T. gamsii* P2-18 sp. removed 91.45 % of glyphosate contaminants leaving 121.83 mg kg^{-1} of AMPA. In addition, it was observed that there was 92.07% glyphosate removal when inoculated with *A. niger* APBSDSF96 leaving 113.53 mg kg^{-1} AMPA. Interestingly, *A. flavusJN*-YG-3-5 utilized 92.86% without accumulation of AMPA; this had the highest extent of degradation.

Overall, an analysis of the degradation efficiency of the fungi strains in glyphosate degradation showed that the isolates were efficient degraders with percentage degradation above 90% (Fig. 5). However, A. *flavus* EFB01 had the poorest percentage degradation (27.17%) indicating poor metabolism of glyphosate. The degradation efficiency of *A. flavusJN*-YG-3-5 was the most efficient fungi (85.60%).

3.5 Molecular characteristics

The molecular characteristics of these promising isolates are shown in Table 4. BLAST analysis (ITS gene sequence) carried out through NCBI GenBank showed that the first two fungi sequences were identified as strains of *T. gamsii* P2-18 (94.57% similarity) and *A. flavus* JN-YG-3-5 (99.28% similarity), respectively. Other isolates were identified as *Aspergillus niger* APBSDSF96 (95.22%) similarity, A*. fumigatus* FJAT-31052 (99.30%) similarity, *A. flavus* EFB01 (99.29%) similarity and *P. simplicissimum* SNB-VECD11G (89.91) similarity. The isolates had high level of GC contents ranging from 53.54% in *P. simplicissimum* SNB-VECD11G to 58.66% in *Aspergillus flavus* JN-YG-3-5 suggesting their potential for environmental management.

Table 4 Molecular characteristics of the isolates

Organisms Name	Identity (%)	E-Value	Sequence length (Bp)	% Guanine-Cytosine
Trichoderma gamsii P2-18	94.57	0.0	589	56.71
Aspergillus flavus JN-YG-3-5	99.28	0.0	612	58.66
Aspergillus niger APBSDSF96	95.22	0.0	590	58.31
Aspergillus fumigatus FJAT-31052	99.30	0.0	583	57.98
Aspergillus flavus EFB01	99.29	0.0	588	56.8
Penicillium simplicissimum SNB-VECD11G	89.91	2E+-147	579	53.54

The ITS gene sequence showed that all the six isolates clustered into three group (*Penicillum sp., Trichoderma sp.* and *Aspergillus sp.)* (Fig. 6) for phylogeny analyses of the isolates. *A. flavus* JN-YG-3-5 clustered with genus *A. flavus* EFB01 showing similarity, they distantly clustered with *A. niger* APBSDSF96 and *A. fumigatus* FJAT-31052. However, *Trichoderma gamsii* P2-18 and *Penicillium simplicissimum* SNB-VECD11G out clustered.

3.6 Fungi genome annotation

Automated annotation identified several genes using a statistical significance threshold (Table 5). The genome sequences of the fungi were compared to those of several organisms (*Archae generic, C. pefringes, B. subtilis* and *P. putida*) known to function in metabolic processes. Validation of the sequence annotation using the FGENESB database yielded the following result: *Rhizobium huautlense* comprises 5 potential protein-coding genes, 1 operon and 4 transcription units. *Pseudomonas aeruginosa strain MZ4A* contains 11 protein genes, 1 operon and 7 transcriptional units. *Pseudomonas aeruginosa* strain *22ABUH7* had 5 protein genes, 1 operon and 3 transcriptional units. *Bacillus subtilis* strain VBN01 had 8 protein genes, 1 operon and 5 transcriptional units. *Pseudomonas aeruginosa* strain HS-38 sequence was made up of 6 potential protein-coding genes, 1 operon and 5 transcriptional units. *P. aeruginosa strain MZ4A* and *P. aeruginosa* strain HS-38 had potential protein-coding genes similar to *P. putida* while others did not. A search of the identified proteins for specific functions revealed that the genes are distributed in different functional categories majorly protein metabolism and respiration (Table 5). Numerous genes associated with pesticide degradation were identified.

Table 5: Gene Statistics using FGENESB

Organism	Closest organism	No of Predicted protein coding genes	No. of Operons	No of Transcript units	Predicted prote
T. gamsii P2-18	*C. pefringes*	4	1	2	MPVRASFQPSI
					P
MTLGQACPPEYWRAQCAFKDSMIH					
MPARILAGAMCVQRFDDSLNSAIHITYRISLRSSSMPEPRDPLLKVLIHFDFLLRAVKIT					
SARGLQ					
LVGPSAAPGSGLRRTRGVTPRAQFGMFTWVGVVTRNDPSAGSQRNLYIFYF					
A. flavus JN-YG-3-5	*C. pefringes*	2	0	2	LRPLVFRGACL
					GAARTQINLTT
MGSNDARTGMPPGIPGGAMCVQRLDDSRNSAIHTSYRISLRSSSMPEPRDPLLKVLTDCD					
TINSDFTRSDRVRGVSGGRGPGAESPRRP					
A. niger APBSDSF96	*C. pefringes*	2	1	1	MVGIRRQAPAN
					PPGIPGGAMCV
					VFVLGSPAGTG
LGPKGPGTRVMILPQGSPTETLGYDF					
A. fumigatus FJAT-31052	*C. pefringes*	2	0	2	MGLCHLLCRPC
VTKPHTLEDRTRCRRCLSGPSPGRGGRGPNTQAVLEGSNDARTGMPPGIPGGAMCVQRLD					
DSLNSAIHITYRISLRSSSMPEPRDPLLKVLTDYDNQLRLHTFRTAFMLGSSAGAGPGAQ					
GLPGGRRNGGPAEATRYDRHGWEVGPTGPSLGNDPSAGSPTETLLRFFTS					
A. flavus EFB01	*C. pefringes*	2	0	2	LRPLVFRGACL
					GACRTQINLST
MGSNDARTGMPPGIPGGAMCVQRLDDSRNSAIHTSYRISLRSSSMPEPRDPLLKVLTDCD					
TINSDFTRSDRVRGVSGGRGPGAESPRRP					
P. simplicissimum SNB-VECD11G	*C. pefringes*	5	2	3	LLYNRLLIGPTE
VPPLPFGPAPREPGGEAQHTSRLEGSNDARTGMPPEIPGGAMGVQRLDVSLISDIQLRID					
FAAFFIEAEQKTVVEV					
VLSYLCSGAPSRPPGASAPGPCREDTNNSVEDAVEQIS					
MPPAAVRGPPNTGTITRGRWPRGLPECPSRSLDGCTNGIPPRPRGPIF					
MHARPTGVQRFRPCLQAPAFRGCHCLVPVFEFAAPPHVRAAIMRRL					

Discussion

Our findings showed that the topsoil from the farms in various locations contains residues of herbicide chemical glyphosate and its metabolite, AMPA. This can be attributed to the over-reliance on this chemical in agricultural practices. However, the concentration of this glyphosate in the field was found to be relatively higher than published work on Environmental Health Criteria 159 under the sponsorship of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization [7]. Therefore, this calls for serious remedial action to be taken as the accumulation of glyphosate is likely to pose a serious danger to ecological receptors.

Notwithstanding the diversity of organisms present in the contaminated site, our interest was majorly on fungi as little is known on their role in the biodegradation of glyphosate. There was high enumeration of fungal count in the location where AMPA level was remarkably high as well as relatively high level of glyphosate compared to lower levels. This was also supported by the Pearson correlation which shows a high correlation with AMPA. A range of fungal strains has been implicated to be abundant in glyphosate contaminated environment either because of their capability of using the compound as the sole source of phosphorus, carbon or nitrogen [20]. As such, they play a role in degradation. Therefore, investigation of the role of diversities of fungi in the degradation of glyphosate can be remarkably interesting.

In order to isolate potential fungi to degrade glyphosate, we observed the enhanced growth of these microbes from the four soil locations. Only six isolates from all the locations demonstrated enhanced growth in the presence of glyphosate. This shows their ability to use glyphosate as an energy source. However,

the inability of the other isolates to survive could be the toxic effect of glyphosate on the organisms. Eman *et al.* [21] noted that the application of pesticides has the possibility to exert some effects on non-target organisms, plus the soil microorganisms. The presence of pesticides makes some microorganisms to lyse while other microorganisms may be resistant and tolerant to a pollutant, hence, an increase in their numbers and biomass due to decreased competition [21].

In studying the effect of glyphosate on the activities of the fungi in the enriched medium supplemented with glyphosate, the pure isolates induced changes in the medium such as changes in pH, optical density, and fungal counts. The decrease in the pH levels of the culture medium may be a result of microbial metabolism and the production of secondary metabolites. Analysis of supernatants by Montserrat *et al.* [22] also demonstrated a decrease in pH resulting from the rapid production of lactic, acetic, pyruvic and citric acids. The implication is that such changes in pH can influence fungi growth. For instance, Yang *et al.* [23] found out that the pH level of the culture medium was one of the key factors influencing the growth of four bacteriocinogenic strains. Furthermore, LeBlanc *et al.* [24] stated that the growth of *Lactobacillus fermentum* CRL 722 was noticeably slower at pH 4.5 (μ_{max} = 0.78 h^{-1}) than at other pH values including pH 5.0, 5.5, and 6.0 (μ_{max} = 1.15 – 1.25 h^{-1}). This agrees with results obtained in this study which shows that optimum growth of fungi under glyphosate was obtained at approximately 5.0. This might also influence bioremediation activity. Therefore, changes in the metabolic state of the fungal can be the driving force of the pH suggesting the active state of the fungal cells. A longer lag phase (16 days after inoculation) could suggest that fungal enrichment in glyphosate treated medium may be slower than bacteria. However, efficiency in the transformation of glyphosate needs to be evaluated. The short exponential phase could suggest an active state of the fungi towards glyphosate remediation. Stratton and Stewart [25] observed a small rise in microbial biomass but no negative or positive effects with respect to the number of microorganisms. In addition, Haney *et al.* [26] and Busse *et al.* [27] assessed the effect of glyphosate on soil's microbial community and their findings concluded that microbial activity was stimulated even in the presence of this herbicide. Therefore, it is prospective that the glyphosate provided nutrients for fungal growth, as shown by the significant growth and increase in the microbial population.

Glyphosate is a nonselective, broad-spectrum, post-emergence herbicide that is widely used in agriculture; hence, degradation of the compound will be a positive obligation in agricultural practices. It was evident that the isolates of fungal strains degraded glyphosate. The growth ability of the fungal strains could ascertain significant assimilation of glyphosate. One reason could be that the fungal strains exhibited optimal growth rates, in order to potentially adapt to the glyphosate concentration and to assimilate it. Also, it is possible that they possess enzymes capable of cleaving the C–P bond. It is remarkable to mention that this assimilation took place without enhancement by sucrose, nitrogen (N), and phosphorus source. For example, Eman *et al.* [21] reported that a concentration of 1% sucrose was important for the initial stimulation of fungal strains in glyphosate degradation. Studies reported that the development of enhanced degradation of xenobiotics or pollutants depends on multiple factors such as nutrient composition, chemical structure, soil properties including the presence of degrading microbes with appropriate metabolic functions [28, 29]. Our aim was to isolate the key player of glyphosate degrading fungi from soils which demonstrated rapid degradation capability. The intensity of glyphosate biodegradation with the indigenous microbial pure strain was highest in *A. flavusJN*-YG-3-5 which utilized 92.86% without accumulation of AMPA. Thus, this makes it so interesting for environmental applications. Other strains had high capability but produced AMPA which might be detrimental to the environment.

Aspergillus spp have received tremendous interest for their suitability in bioremediation [30]. This could be the reason scientists and environmentalists are interested to develop various strategies for the use of *Aspergillus* sp. in bioremediation. This species will be useful in pesticide-contaminated soil. Different species of fungi were identified using BLAST analysis. The high abundance of *Aspergillus* species in the samples may be due to their ability to tolerate and degrade pesticides. Similar studies have been conducted by Asef, [14], and have revealed the isolation, molecular characterization, and pesticide degradation by *Aspergillus* species. Thus the reason *Aspergillus sp* have received tremendous interest in suitability to remediate a wide range of xenobiotic compounds. The identified fungal strains observed in this study have high GC contents. This is likely to have made them tolerant of pesticides. One imperative property of the GC base pair is its higher thermal stability than the AT base pair. An increase in GC content correlates with a broader tolerance range of species [31].

The range of GC contents in the fungi suggests characteristics of microbe from the soil. *Aspergillus flavus JN-YG-3-5* can be a particularly important tool for use in biotechnology because it yielded high pure DNA quantity and has a GC content similar to well-known GC in the soil for active physiological functions. The works of Smarda *et al* [31] and Njoku *et al.* [18] reported that GC-rich genes facilitate the response to environmental stress. In addition, it can also facilitate complex gene regulation. Thus, improved responses to environmental conditions might be enabled by GC-rich genes. The fungi having higher GC contents were better in glyphosate degradation thus giving a beneficial advantage to be utilized in a wide range of environmental applications. This could have also been an added advantage to *Aspergillus flavus* JN-YG-3-5 for the complete mineralization of glyphosate pesticide.

Phylogenetic analysis explicitly showed that the polluted soil sheltered diverse fungi population belonging to three clusters of orthologous groups with *Aspergillus flavus* JN-YG-3-5 clustering together suggesting their similar ancestry. It can also be due to a combination of selective factors, proximity and functional capacity [32]. The different groups that they belong to do not necessarily mean that they degraded the contaminant through different processes. It has been hypothesized that phylogenetically distant lineages might share mutual functions and functional features. The work agreed with the work of Ning and Beiko [32] who reported that functional similarities exist between operational taxonomic units (OTUs) that belong to different high-level taxonomic groups for fungi.

Automated annotation identified several proteins within the genome of fungal strains to include ATP-binding cassette (ABC) transporters, these are members of a protein superfamily known to be involved in the efflux of drugs from the cells of target organisms. Also, the Zinc finger protein gene and zinc finger chimera 1, were discovered along with many oxidoreductase genes. A search of the identified proteins for specific functions revealed that the genes are distributed in different functional categories majorly protein metabolism and respiration. Numerous genes associated with pesticide degradation were identified.

Interestingly, The GhostKOALA output identified the CYP2W1 gene (Cytochrome P450, fungi type) present in *Aspergillus fumigatus* strain FJAT-31052 which was absent in the genome of other fungi. The cytochrome P450 enzymes are monooxygenases that catalyze many active reactions involved in the metabolism of a wide variety of xenobiotics [33]. Previous studies have reported the activities of human CYPs involved in the metabolism of pesticides [34, 35]. CYP-pesticides interactions are by either the induction or inhibition of the metabolizing enzymes. In a study by Khaled *et al.* [33] HepaRG cells express a large panel of liver-specific genes including several CYP enzymes, which contrasts with HepG2 cell lines. Both immunoblotting and reverse transcription polymerase chain reaction (RT-PCR) techniques have been used to examine the pesticide-CYP induction [33, 36, 37].

Although fungi have received tremendous interest for their suitability in detoxifying a variety of contaminants, its ability to degrade glyphosate is a new area of research interest. Two different routes have been proposed to be utilized by soil microorganisms to metabolize glyphosate: The C-P lyase and AMPA pathways [9]. To demonstrate the valid pathway, identification of AMPA even to a significant amount shows that AMPA pathway is valid. This mechanism involves the oxidative cleavage of the C-N bond on the carboxyl side catalyzed by glyphosate oxidoreductase (GOX) which results in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate. The mechanism for detoxification of glyphosate was suggested by activities of certain enzymes that catalyze the reaction such as oxidoreductases that cleave C–N with the stoichiometric formation of glyoxylate and aminomethylphosphonic acid (AMP) [6]. Aminotransferase catalyzes the conversion of AMP to phosphonoformaldehyde. Phosphonatase catalyzes the cleavage of phosphonoacetaldehyde C–P bond to form acetalde-hyde. It was evident and validated from the annotated gene results that the dehydrogenase/oxidoreductase related pathway is valid by the presence of dehydrogenase related protein (alcohol dehydrogenase) discovered in their genome.

Conclusion

In this study, novel glyphosate-degrading fungi strains were isolated from farm soils in Nigeria. All strains used for enhanced biodegradation grew in the presence of glyphosate and were able to degrade glyphosate. This is the first report to show the fungal degradation of glyphosate from Nigerian soil. The use of these indigenous fungal strains promises to be effective in the practical application of bioremediation of glyphosate since the microbes have already adapted to the localized habitat conditions. The essence of this is that isolated strains can also be added to other soils as microbial inoculants for their potential to degrade pesticides by improving soil quality for sustainable agriculture and the environment. This study has provided strains with biodegrading genes, enzymes and pathways to be harnessed for a range of biotechnological and bioremediation applications. It provides novel insights into specialized organisms for active bioremediation. The physiological and molecular characteristics show that *Aspergillus* species are useful organisms for managing contamination by glyphosate pesticide.

Declarations

Availability of data and materials

All generated or analysed data during this study are included in this published article and are also available from the corresponding authors upon request.

Competing interests

The authors declare they have no competing interests.

Funding

Not applicable.

Authors' contributions

KLN, EOU and EOP carried out the design and development of the research idea. EOP and EOU carried out the biological and technical experiments. AOO, AAA and KLN carried out formal analyses and review. All the authors read, agreed and approved the final manuscript.

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Figures

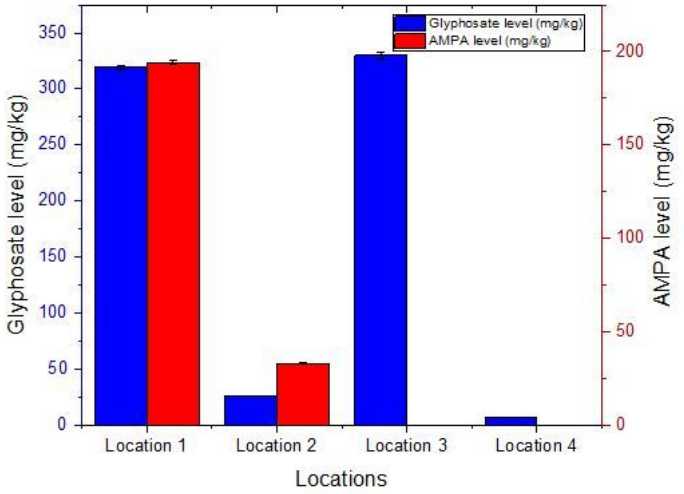

Figure 1

The initial glyphosate residue of the different soil locations (left) and transformation product AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) (right). Values are mean levels with standard errors of the pollutants in the locations where fungi were isolated.

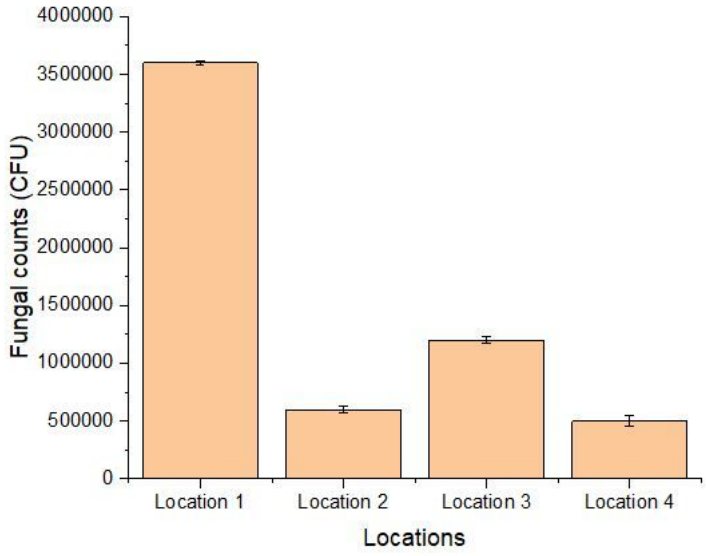

Figure 2

Fungal counts (CFU) of soil samples from the different locations. The values are means of the fungal counts with standard errors isolated from the farms where glyphosate is used as pesticide.

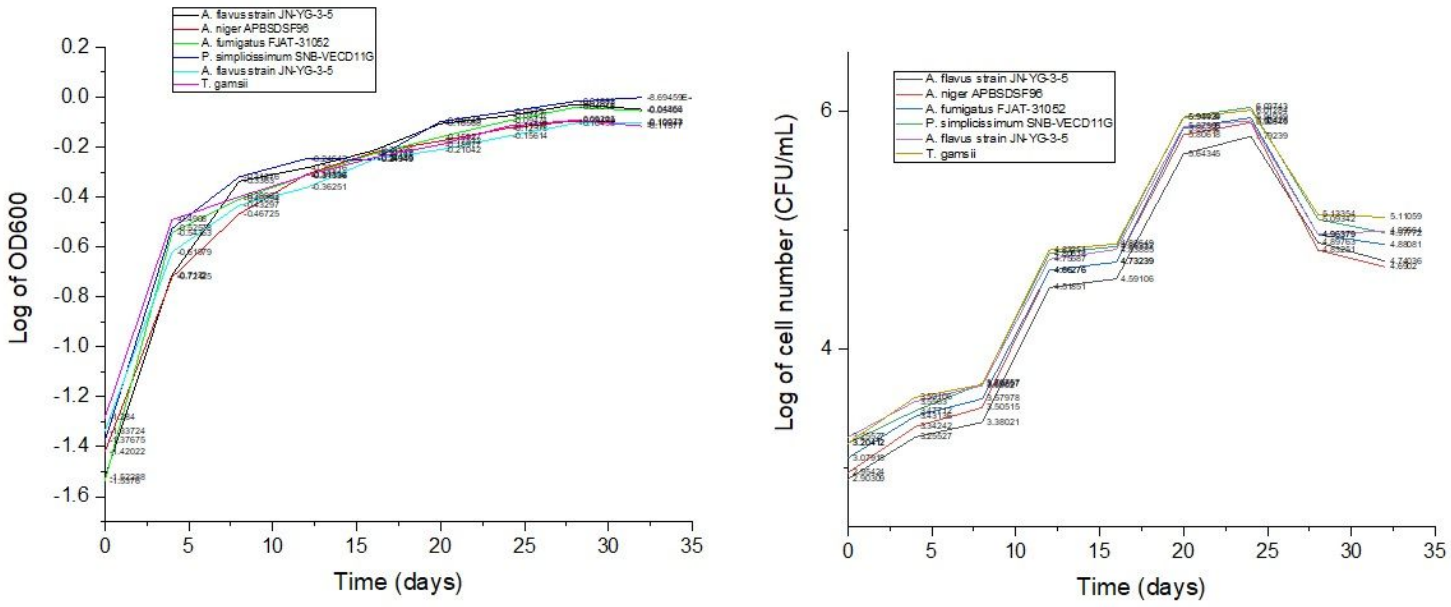

Figure 3

Time dependent changes in microbial growth of the different fungal isolates over the course of 32 d of incubation. (a) OD600 of 1 mL aliquots of fungal cultures were measured using a spectrophotometer. (b) Cell number (cfu/mL) was done microscopically every four days for 32 d.

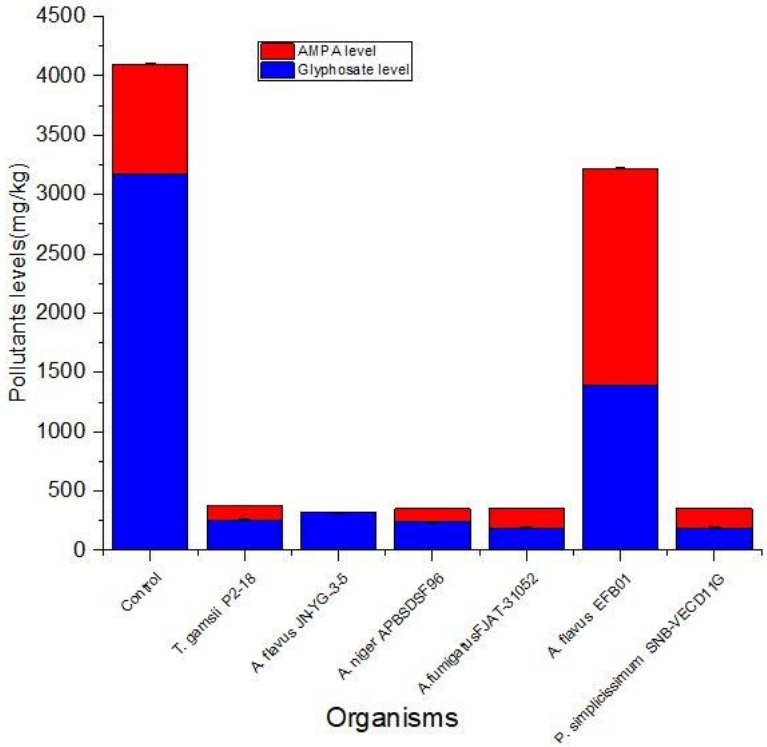

Figure 4

Glyphosate biodegradation by the various organisms

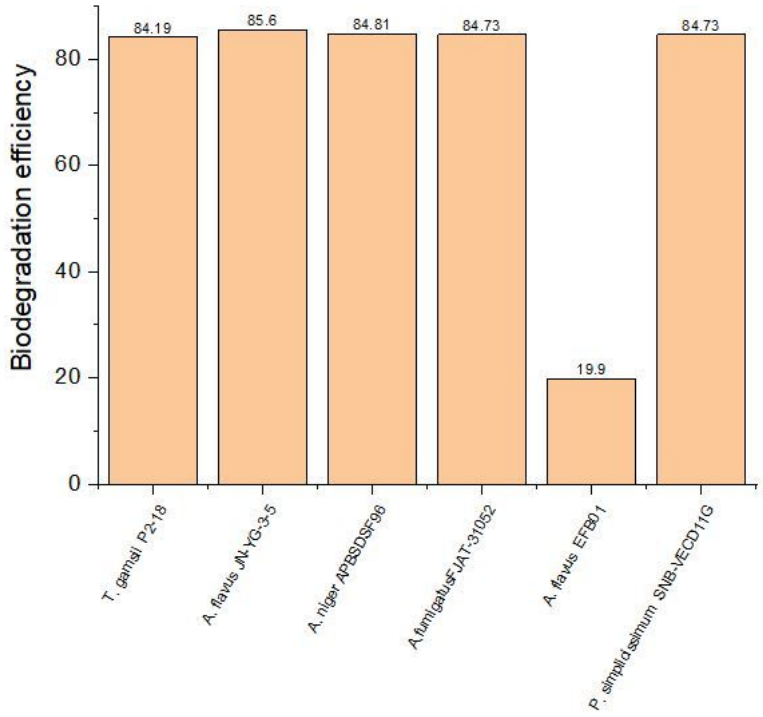

Figure 5

Degradation coefficient of glyphosate by the various organisms

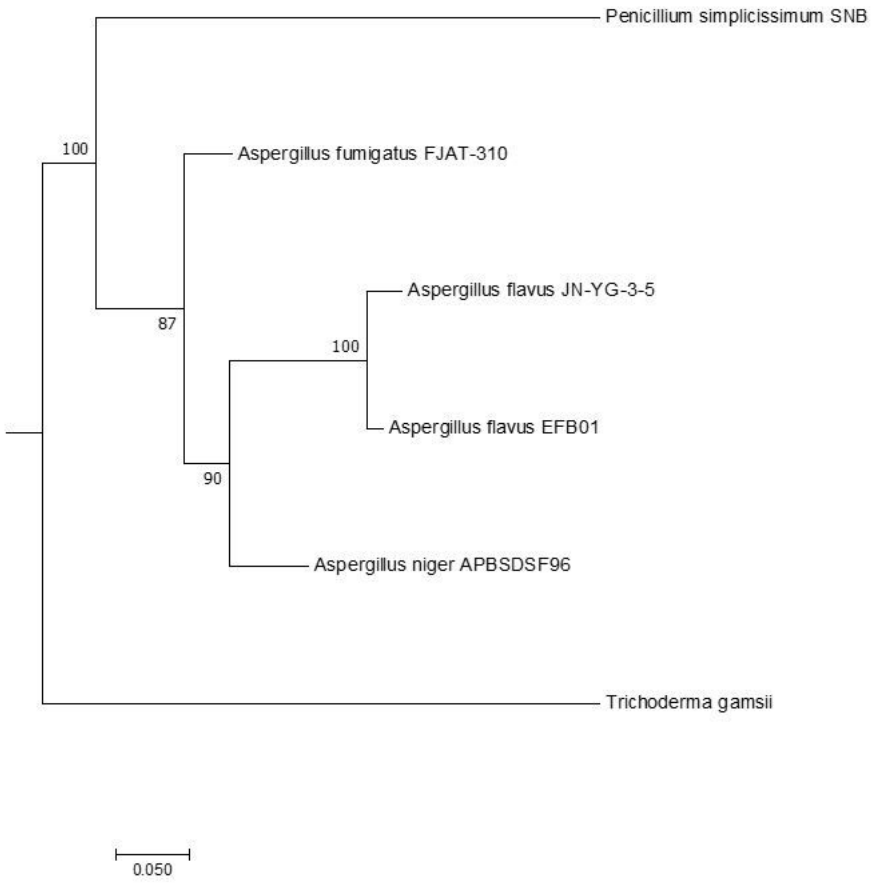

Figure 6

Phylogenetic relationship of fungal isolates

Supplementary Files

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